

Development and Optimization of a Novel Immunomagnetic Separation-Bacteriophage Assay for Detection of *Salmonella enterica* Serovar Enteritidis in Broth

STACY J. FAVRIN,¹ SABAH A. JASSIM,^{1†} AND MANSEL W. GRIFFITHS^{1,2*}

Department of Food Science¹ and Canadian Research Institute for Food Safety,²
University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received 14 June 2000/Accepted 13 October 2000

***Salmonella* is the second-leading cause of food-borne illness in most developed countries, causing diarrhea, cramps, vomiting, and often fever. Many rapid methods are available for detection of *Salmonella* in foods, but these methods are often insensitive or expensive or require a high degree of technical ability to perform. In this paper we describe development and characterization of a novel assay that utilizes the normal infection cycle of bacteriophage SJ2 for detection of *Salmonella enterica* serovar Enteritidis in broth. The assay consists of four main stages: (i) capture and concentration of target cells by using immunomagnetic separation (IMS); (ii) infection of the target bacterium with phage; (iii) amplification and recovery of progeny phage; and (iv) assay of progeny phage on the basis of their effect on a healthy population of host cells (signal-amplifying cells). The end point of the assay can be determined by using either fluorescence or optical density measurements. The detection limit of the assay in broth is less than 10⁴ CFU/ml, and the assay can be performed in 4 to 5 h. The results of this study demonstrate that the IMS-bacteriophage assay is a rapid, simple, and sensitive technique for detection of *Salmonella* serovar Enteritidis in broth cultures which can be applied to preenriched food samples.**

Salmonellae remain a major cause of food-borne illness in the developed world, causing gastroenteritis characterized by diarrhea, cramps, vomiting, and often fever. *Salmonellae* are ubiquitous in the environment and primarily reside in the intestinal tracts of birds, reptiles, farm animals, and humans. Transmission to humans is usually food borne and results from eating undercooked meat, milk, or eggs or from cross-contamination of other foods which are eaten without cooking.

Conventional methods for detection of *Salmonella* in foods are based on nonselective and selective enrichment, followed by biochemical and serological identification. These methods can take from 4 to 6 days to perform and thus lack the speed required for analysis of food products. Numerous rapid methods have been developed for *Salmonella* detection, but these methods often suffer from a lack of sensitivity or specificity or require expensive equipment or considerable technical expertise to perform (3, 22, 30).

The specificities of bacteriophages for their host bacteria make them ideal agents for bacterial identification and strain typing (7). Bacteriophages (phages) have been investigated for use in detecting food-borne pathogens. Bacterial luciferase has been used as a reporter for bacterial detection and enumeration (28). To do this, *lux* genes are introduced into the genome of a bacteriophage. The bacteriophage lacks the intracellular machinery necessary for light production and thus remains dark. Upon infection of a host, the phage genes, including the additional *lux* genes, are expressed within 1 h of infection and the host cells become bioluminescent (28). Kodikara et al. (14) have used

lux⁺ recombinant phage for nearly on-line detection of enteric indicator organisms. If a target is present at levels greater than 10⁴ CFU/g, it can be detected without enrichment in less than 1 h. Chen and Griffiths (5) used *lux*⁺ recombinant bacteriophage for detection of *Salmonella* in eggs. Recombinant transducing phage were introduced into artificially inoculated eggs. Infection of target *Salmonella* by the phage resulted in luminescence, which could be detected directly through the eggshell with a charge coupled device photon-counting camera. A 6-h preenrichment period was sufficient for detection of as few as 10 *Salmonella* cells per ml of original sample. An advantage of these luminescent applications is the sensitivity of the instruments available to detect and quantify light output. The Bacterial Ice Nucleation Diagnostic (BIND) test (Idetek Corp.) is based on production of ice nuclei upon infection by phage, which results in ice crystal formation in supercooled water (32). A major limitation of such methods is the genetic manipulation required to prepare the bacteriophage for the test.

Immunomagnetic separation (IMS) has been used as an alternative to selective enrichment broths for a variety of bacteria, including *Salmonella* spp. Paramagnetic beads are coated with polyclonal antibodies, which can target and separate *Salmonella* cells in a mixed suspension with no loss of viability, producing a normal isolate for further confirmation. IMS can eliminate the need for selective enrichment and reduce the time required for conventional methods by as much as 24 h (15). IMS has also been used in conjunction with other rapid detection methods, including enzyme-linked immunosorbent assays (ELISAs), conductance microbiology, electrochemiluminescence, and PCR (6, 8, 12, 20, 34).

The objective of this study was to develop a bacteriophage-based assay to detect *Salmonella enterica* serovar Enteritidis in a timely, inexpensive, and technically simple manner. The assay developed capitalized on the inherent specificity of a bac-

* Corresponding author. Mailing address: Department of Food Science, University of Guelph, Guelph, ON, Canada N1G 2W1. Phone: (519) 824-4120, ext. 2269. Fax: (519) 763-0952. E-mail: mgriffith@uoguelph.ca.

† Present address: Zayed Complex for Herbal Research and Traditional Medicine, Ministry of Health, Abu Dhabi, United Arab Emirates.

terioophage for its target bacterium and was based on the normal infection cycle of the bacteriophage in its host; thus, it required no genetic manipulation. In addition, the assay detected only viable bacteria, leaving target bacteria killed by disinfectants or other bactericidal treatments undetected.

MATERIALS AND METHODS

Media. L broth (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], and 5 g of sodium chloride [Fisher Scientific, Whitby, Ontario, Canada], in 1 liter of distilled water; pH 7.2) was used throughout the protocols where indicated. L agar consisted of L broth plus 10 g of agar (Difco) per liter and was used for culture maintenance. IMS washing steps were carried out with lambda buffer (2.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.006 g of gelatin, and 6 ml of 0.1 M Tris buffer [pH 7.2] in 1 liter of distilled water). For plaque assays, the top layer agar consisted of 10 g of tryptone per liter, 5 g of yeast extract (Difco) per liter, 5 g of sodium chloride (Fisher Scientific) per liter, and 4 g of agar (Difco) per liter.

Bacterial strains. A *Salmonella* serovar Enteritidis strain was isolated from an egg in our laboratory and designated strain C721. This strain was used throughout development of the method described here. The strain was maintained on L-agar plates and transferred bimonthly. Fifty-five bacterial isolates were used for the specificity studies. Table 1 shows the 20 strains of *Salmonella* serovar Enteritidis, 29 strains of non-serovar Enteritidis salmonellae, and six strains of non-*Salmonella* bacteria used for the specificity study. Fifteen of the serovar Enteritidis isolates were from food and human sources obtained from the Laboratory Center for Disease Control, Ottawa, Canada, and all were from a single outbreak.

Bacteriophage. The bacteriophage used in this study, bacteriophage SJ2, is a lytic phage isolated in our laboratory from an egg. Bacteriophage was propagated by using the plate method in the host *Salmonella* serovar Enteritidis C721. One hundred microliters of phage and 100 μl of an overnight host culture were added to 2.5 ml of top layer, and the agar was cooled to 45°C and poured over L-agar plates. The plates were incubated overnight at 37°C. Phage were recovered from the plates by using lambda buffer as the recovery medium. Lysate was treated with chloroform at a ratio of chloroform to lysate of 1:3 for 10 min and centrifuged for 15 min at 5,000 \times g with a Beckman G2-MC centrifuge (Beckman Coulter Inc., Fullerton, Calif.). Supernatant was recovered and syringe filtered through a 0.2- μm -pore-size syringe filter (Nalgene, Rochester, N.Y.). The bacteriophage suspension was stored at 4°C.

One-step growth experiment. In order to characterize phage SJ2, a one-step growth experiment was performed to determine (i) the burst time (the latent period between adsorption and lysis) and (ii) the burst size (the average number of phage particles released per infected host cell). The method used was adapted from the method of Maramorosh and Koprowski (16). Briefly, the phage were allowed to infect a host strain preparation, which was then greatly diluted to halt attachment. The infection cycle was continued with frequent plaque assays of samples.

Bacteriophage specificity. Bacteriophage SJ2 was tested with 55 bacterial strains (Table 1) to determine its specificity. Plaque assays were performed based on a modified method for quantitative assay of phage described by Maramorosh and Koprowski (16). A phage SJ2 suspension (100 μl) and 100 μl of the overnight culture to be tested were added to 2.5 ml of molten top layer agar cooled to 45°C. The mixture of phage, cells, and agar was quickly poured onto 10-cm-diameter petri plates (Fisher Scientific) containing hardened L agar, and the plates were swirled to cover their surfaces. When the top agar layer had solidified, the plates were incubated overnight at 37°C and then examined for the presence of plaques.

Principles of the IMS-bacteriophage assay. A flowchart of the IMS-bacteriophage protocol is shown in Fig. 1.

(i) Capture. Twenty-microliter portions of anti-*Salmonella* Dynabeads (DynaL, Lake Success, N.Y.) were added to Eppendorf tubes containing 1-ml portions of a stationary-phase culture of *Salmonella* serovar Enteritidis. The tubes were rotated at 30 rpm for 30 min at room temperature on an Orbitron Rotator II (Fisher Scientific), and then samples were placed in a magnetic particle separator (Boehringer, Mannheim, Germany) for 3 min in order to separate the magnetic beads from the broth. The beads were washed twice in lambda buffer and resuspended in 250 μl of L broth.

(ii) Attachment and amplification. One hundred microliters of phage was added to each sample, and the samples were incubated for 10 min at 37°C to allow attachment. The magnetic beads were separated and washed twice in lambda buffer to remove unbound phage. The beads were resuspended in 100 μl of L broth and incubated for 30 min at 37°C to allow release of progeny phage.

(iii) SAC incubation. The magnetic beads were captured, and the progeny phage recovered in the supernatant were added to a plastic cuvette containing 1 ml of signal-amplifying cells (SACs) in L broth. SACs were prepared by diluting

an overnight culture of *Salmonella* serovar Enteritidis in L broth to an optical density at 600 nm of 0.075. Absorbance at 600 nm was determined with a Pharmacia Novaspec II spectrophotometer (Amersham Pharmacia Biotech Inc., Uppsala, Sweden). Negative control samples, consisting of 1 ml of L broth, were tested in duplicate alongside positive samples.

(iv) End point detection. The progeny phage and SACs were incubated for 1.5 to 2 h at 37°C. The optical densities of samples were determined. The optical densities of the contents of duplicate sample tubes were calculated as percentages of the mean negative control value. A test which resulted in a value of 70% or less of the mean negative control value was considered a positive test.

(v) Alternative end point detection. Alternatively, SACs were incubated in Eppendorf tubes, and following 1.5 h of incubation, samples were centrifuged at 14,000 rpm for 10 min with an Eppendorf 5415 bench top centrifuge (Brinkmann Instruments Inc., Westbury, N.Y.). The supernatant was discarded, and each pellet was resuspended in 900 μl of lambda buffer. Samples were stained with 100 μl of LIVE/DEAD BacLight (Molecular Probes, Eugene, Oreg.) bacterial viability stain solution. The dye solution was prepared so that a 100- μl portion contained 1.5 μl of the live stain (component A, SYTO 9) and 1.5 μl of the dead stain (component B, propidium iodide) in lambda buffer. Two-hundred-microliter portions were distributed in quadruplicate into clear 96-well microtiter plates. A blank consisting of the dye and lambda buffer was also included. Samples were left in the dark for 15 min, and green fluorescence was read with an FL500 fluorometer (Bio-Tek Instruments, Winooski, Vt.). The excitation wavelength was 485 nm, and the emission wavelength was 510 nm. Sample values were calculated by determining the mean of four replicate fluorescence readings for each tube. Values for duplicate sample tubes were calculated as percentages of the mean negative control value. A test which resulted in a value of 70% or less of the mean negative control value was considered a positive test.

Efficiency of IMS. To test the efficiency of magnetic capture, an overnight culture of *Salmonella* serovar Enteritidis was serially diluted 10-fold in L broth to a dilution of 10^{-6} . The cell concentrations in the six dilutions were estimated to be 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , and 10^3 CFU/ml. Immediately after dilution, the efficiency of magnetic capture was tested with 1 ml of each dilution in duplicate. To determine the cell populations in the initial dilutions, duplicate spread plate counts were determined. Magnetic capture and washing were performed as described above. The beads were resuspended in 1 ml of lambda buffer and vortexed, and duplicate spread plate counts for sample were determined on L-agar plates. Serial dilutions were made as required to enumerate the organisms in samples containing high numbers of cells. All plates were incubated overnight at 37°C. Colonies were counted, and the resulting IMS plate counts were compared to the numbers of CFU per milliliter in the original dilutions.

Assay parameters. **(i) Temperature.** The IMS-bacteriophage assay was tested at three temperatures (30, 37, and 42°C) to determine the optimum temperature for the assay. Each incubation period (attachment, amplification, and SAC incubation) was carried out at each experimental temperature. Positive and negative samples were tested in duplicate at each experimental temperature.

(ii) Phage population. The IMS-bacteriophage assay was performed by using three phage populations (10^6 , 10^7 , and 10^8 PFU/100 μl) with a *Salmonella* serovar Enteritidis population containing 10^6 CFU/ml in order to determine the optimum phage population for the assay. Positive and negative samples were tested in duplicate for each phage population in each of two trials.

(iii) Media. Preliminary fluorescence studies showed that the L broth used for the final incubation step in the assay interfered with the fluorescent dye, causing high background readings. Thus, a centrifugation step was included in order to wash the cells and remove the medium residue prior to fluorescent staining. An alternative to L broth which would not interfere with fluorescent staining was sought for the SAC incubation step in order to eliminate the washing step. L broth was compared to lambda buffer, as well as to lambda buffer supplemented with two sugars, dextrose and maltose, at three concentrations (0.2, 0.5, and 1.0%). Each of the media was compared to L broth to determine its ability to support growth of SACs and provide a suitable environment for reduction of SACs by phage.

(iv) Freeze-dried SACs. One hundred microliters of an overnight culture was transferred to 30 ml of L broth and incubated for 8 h at 37°C with shaking. Thirty-milliliter portions were centrifuged for 10 min at 5,000 \times g. The supernatant was discarded, and each pellet was resuspended in 30 ml of lyophilization medium (50 g of dextran per liter, 75 g of sucrose per liter, and 100 g of sodium glutamate per liter in distilled water). Two-hundred-microliter portions were distributed into sterile 2-ml serum vials (Fisher Scientific), and the vials were loosely closed with butyl slotted-plug stoppers (Fisher Scientific). The vials were frozen at -65°C overnight and then transferred to a -70°C freezer for 4 h. The vials were freeze-dried with a Lyph-lock 6-liter freeze-dryer (Labconco, Kansas City, Mo.) equipped with a stoppering tray dryer (model 7756; Labconco) for

TABLE 1. Bacterial strains used for the specificity study and results of the IMS-bacteriophage assay

Serogroup	Taxon	Strain	Origin ^a	Plaque assay results/IMS assay results
D	<i>Salmonella</i> serovar Enteritidis	C721	Egg	+/+
	<i>Salmonella</i> serovar Enteritidis	SA 923451	Health Canada	+/+
	<i>Salmonella</i> serovar Enteritidis	ATCC 13076	ATCC	+/+
	<i>Salmonella</i> serovar Enteritidis	SA 942451	Health Canada	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 2588	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5671	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5713	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5711	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5712	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5493	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 4673	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5670	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5540	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5725	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5523	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5726	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5685	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5612	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5590	LCDC	+/+
	<i>Salmonella</i> serovar Enteritidis	EN 5680	LCDC	+/+
B	<i>Salmonella</i> serovar Berta	SA 941123	Health Canada	+/+ ^b
	<i>Salmonella</i> serovar Panama	SA 931592	Health Canada	+/+ ^b
	<i>Salmonella</i> serovar Sendai	S-513	LCDC	+/+
	<i>Salmonella</i> serovar Typhimurium	SA 941256	Health Canada	+/+
	<i>Salmonella</i> serovar Typhimurium	94-51	LCDC	-/-
	<i>Salmonella</i> serovar Heidelberg	SA 941270	Health Canada	-/-
	<i>Salmonella</i> serovar Saintpaul	SA 941270	Health Canada	-/-
	<i>Salmonella</i> serovar Bredeney	SA 941247	Health Canada	-/-
	<i>Salmonella</i> serovar Schwartzengrund	S-1303	LCDC	-/-
	<i>Salmonella</i> serovar Schwartzengrund	SA 941254	Health Canada	-/-
C	<i>Salmonella</i> serovar Agona	SA 941132	Health Canada	-/-
	<i>Salmonella</i> serovar Indiana	SA 931221	Health Canada	-/-
	<i>Salmonella</i> serovar Brandenburg	SA 941266	Health Canada	-/-
	<i>Salmonella</i> serovar Reading	SA 940082	Health Canada	-/-
	<i>Salmonella</i> serovar Infantis	SA 941223	Health Canada	-/-
	<i>Salmonella</i> serovar Thompson	SA 941182	Health Canada	-/-
	<i>Salmonella</i> serovar Mbandaka	SA 941201	Health Canada	-/-
	<i>Salmonella</i> serovar Braenderup	SA 940524	Health Canada	-/-
	<i>Salmonella</i> serovar Montevideo	SA 9411253	Health Canada	-/-
	<i>Salmonella</i> serovar Ohio	SA 941244	Health Canada	-/-
	<i>Salmonella</i> serovar Oranienburg	SA 940883	Health Canada	-/-
	<i>Salmonella</i> serovar Tennessee	SA 941191	Health Canada	-/-
	<i>Salmonella</i> serovar Johannesburg		Health Canada	-/-
	<i>Salmonella</i> serovar Urbana	SA 921695	Health Canada	-/-
	<i>Salmonella</i> serovar Rubislaw	SA 914038	Health Canada	-/-
	<i>Salmonella</i> serovar Hadar	SA 941264	Health Canada	-/-
	<i>Salmonella</i> serovar Kentucky	SA 941116	Health Canada	-/-
NA ^c	<i>Salmonella</i> serovar Newport	SA 932663	Health Canada	-/-
	<i>Salmonella</i> serovar Choleraesuis	S-870	LCDC	-/-
	<i>Serratia marcescens</i>	ATCC 8100	ATCC	-/-
	<i>Klebsiella pneumoniae</i>	ATCC 1388	ATCC	-/-
	<i>Citrobacter freundii</i>	Laboratory	University of Guelph ^d	-/-
	<i>Shigella flexneri</i>	Laboratory	University of Guelph ^d	-/-
	<i>Escherichia coli</i>	ATCC 25922	ATCC	-/-
	<i>Escherichia coli</i> O157:H7	EC 920333	Health Canada	-/-

^a ATCC, American Type Culture Collection; LCDC, Laboratory Center for Disease Control.^b Weak lysis/not consistently positive as determined by the IMS assay.^c NA, not applicable.^d Department of Food Science.

22 h at a vacuum pressure of 1.5×10^{-1} Pa. The vials were closed with stoppers under a vacuum prior to removal from the chamber. To determine the initial titer of bacteria prior to freeze-drying, 200 μ l of each culture to be freeze-dried was added to 800 μ l of lambda buffer. Tenfold serial dilutions were prepared, and duplicate spread plates were incubated overnight at 37°C. The recovery of cells

following the freeze-drying procedure was determined by resuspending the contents of duplicate vials containing each freeze-dried culture in 1 ml of L broth. Samples were serially diluted 10-fold in lambda buffer, and 100- μ l portions were spread plated in duplicate on L-agar plates. The plates were incubated overnight at 37°C, and plate counts were compared to the titer prior to freeze-drying. In

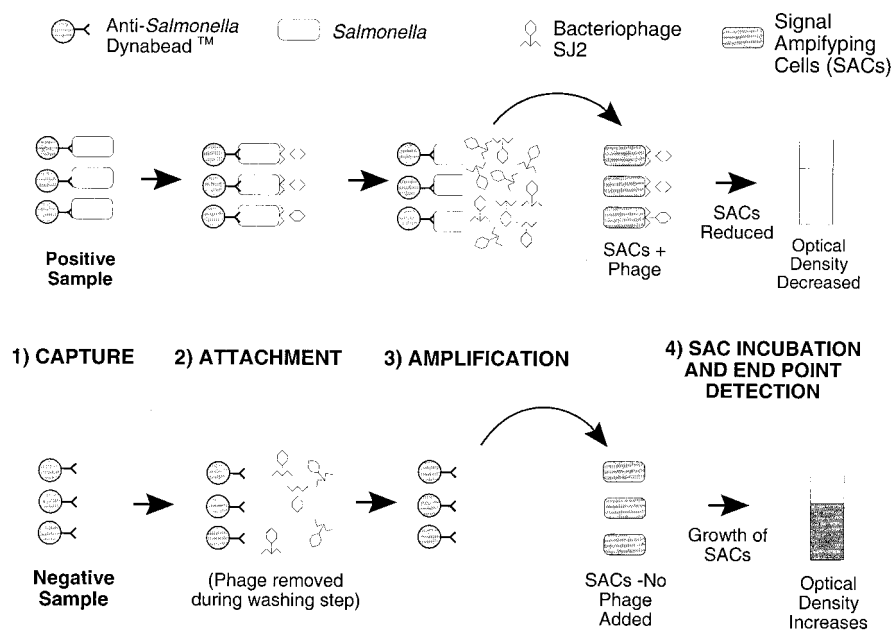


FIG. 1. Flowchart of the IMS-bacteriophage protocol for detection of *Salmonella* serovar Enteritidis. *Salmonella*-positive and -negative scenarios are given. In step 1, *Salmonella* cells are captured and concentrated by IMS with anti-*Salmonella* Dynabeads. In step 2, phage are added to samples and incubated to allow attachment; exogenous phage are removed by washing the beads. In step 3 phage are amplified in the host and are released upon lysis; progeny phage are recovered and added to a healthy population of *Salmonella* serovar Enteritidis cells (SACs). In step 4, the assay end point is determined by measuring optical density (as shown) or fluorescence. Positive samples are identified based on decreased signals compared to the signals obtained for negative control samples.

addition, reconstituted samples were transferred to 1-ml cuvettes and incubated at 37°C. The optical density at 600 nm was monitored over time to determine the length of the lag phase of the cultures.

Sensitivity tests. The limit of detection of the IMS-bacteriophage assay was determined by serially diluting a pure overnight culture of *Salmonella* serovar Enteritidis to concentrations ranging from 10^2 to 10^6 CFU/ml. The IMS-bacteriophage assay was carried out as described above, and each dilution was tested in duplicate in two trials.

An IMS-bacteriophage assay sensitivity test was also performed as described above by using the freeze-dried SAC population. Freeze-dried cultures were resuspended in 1-ml portions of L broth at the start of the protocol and incubated at 37°C until they were required for the assay (approximately 2.5 h). One-hundred-microliter portions of the reconstituted samples were added to 0.9-ml portions of L broth in plastic cuvettes for use as SACs. The optical density at 600 nm of each of these samples was about 0.060.

In order to demonstrate the utility of fluorescence as a suitable end point for the assay, the IMS-bacteriophage assay was performed with five concentrations of an overnight culture of *Salmonella* serovar Enteritidis by using fluorescence measurements as described above to determine the assay end point.

Statistical analysis. Linear regression of plate counts versus IMS plate counts was performed by using Quattro-Pro (Corel Corporation, Ottawa, Ontario, Canada). All statistical analyses involving treatment comparisons were performed by using a one-way analysis of variance at the $\alpha = 0.05$ significance level in Quattro-Pro (Corel). When statistically significant differences existed, post hoc analysis was performed by using Scheffe's contrast test (26).

RESULTS

Specificity study. A plaque assay, as well as the IMS-bacteriophage protocol, was performed with each of 55 bacterial isolates (Table 1) in order to determine the specificity of the phage and the assay. The bacteriophage was broadly specific for the serogroup D *Salmonella* strains tested and did not cross-react with any of the other *Salmonella* strains tested except *S. enterica* serovar Typhimurium SA 941256. This *Salmonella* serovar Typhimurium isolate was confirmed by serology (Difco) to belong to serogroup B.

One-step growth experiment. The propagation curve for phage SJ2 (Fig. 2) shows a burst time of between 32 and 40 min with a burst size of about 245 PFU. Calculation of the percentage of absorption indicated that more than 97% of the phage adsorbed to the host cells during the first 4 min.

IMS-bacteriophage assay. The IMS-bacteriophage assay was performed with six dilutions of *Salmonella* serovar Enteritidis to determine the detection limit in broth. For each sample, an absorbance value was calculated as a percentage of the mean of the negative control values for that trial. A sample value of 70% of the mean negative control value was used as

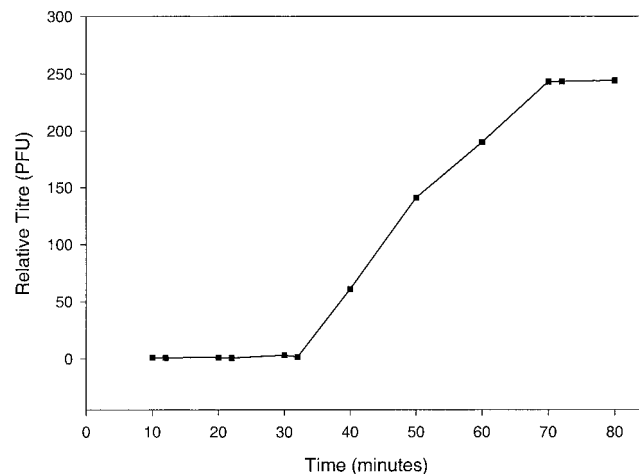


FIG. 2. One-step growth curve for phage SJ2 in *Salmonella* serovar Enteritidis C721. The lag time is approximately 32 to 42 min with a burst size of about 245 PFU.

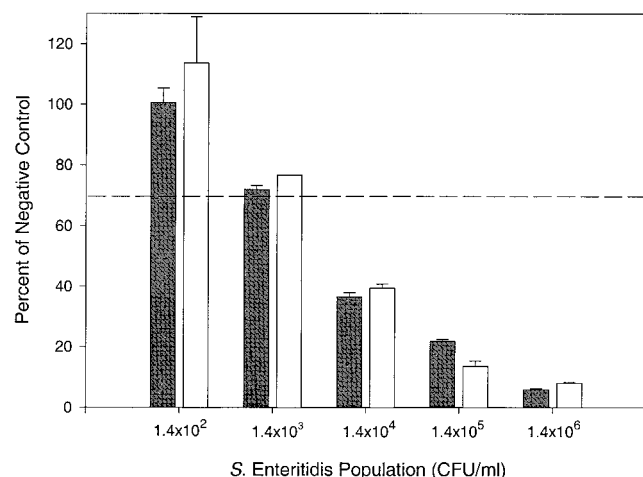


FIG. 3. Sensitivity of the IMS-bacteriophage assay as determined with five populations of *Salmonella* serovar Enteritidis. Values are expressed as percentages of the mean negative control value. The assay end point was determined by using optical density measurements obtained at 600 nm. The dark shaded bars show the results of assays performed with SACs from an overnight culture; the light shaded bars show the results of assays performed with freeze-dried SACs. The dashed line indicates the 70% cutoff value for positive tests. The error bars indicate 1 standard deviation.

the cutoff value for a positive test. This value was arrived at based on preliminary work performed with artificially inoculated food samples and provided reliable discrimination between positive and negative samples. Figure 3 shows that the detection limit was less than 10^4 CFU/ml when either a freeze-dried SAC culture or an overnight SAC culture was used.

The IMS-bacteriophage assay was also performed with five dilutions of *Salmonella* serovar Enteritidis to demonstrate the utility of fluorescence as a way to determine the assay end point. For each sample, the fluorescence value was calculated as a percentage of the mean of the negative control values for that trial. Figure 4 shows that the detection limit was less than 10^4 CFU/ml when fluorescence was used as an end point, confirming the observations made when optical density was used as the end point.

Efficiency of IMS. Plate counts following IMS were compared to spread plate counts for six dilutions of *Salmonella* serovar Enteritidis. The results are shown in Fig. 5. There was a significant positive correlation between plate counts and IMS plate counts ($n = 11$, $r^2 = 0.99$, $P < 0.001$).

Assay parameters. (i) Temperature. The IMS-bacteriophage assay was performed at 30, 37, and 42°C. There was no significant difference in SAC reduction between 37 and 42°C, but these temperatures were significantly better than 30°C ($P < 0.05$). As a result, 37°C was used for the assay.

(ii) Phage population. Three phage populations (10^6 , 10^7 , and 10^8 PFU/100 μ l) were tested in duplicate in two trials to determine the optimum phage concentration for the assay. There was no significant difference between the assay outcomes when 10^7 and 10^8 PFU/100 μ l were used, but these phage concentrations performed significantly better than 10^6 PFU/100 μ l ($P < 0.05$). Efficient removal of unbound phage after the attachment step is very important to the success of the protocol. As a result, 10^7 PFU/100 μ l was considered the op-

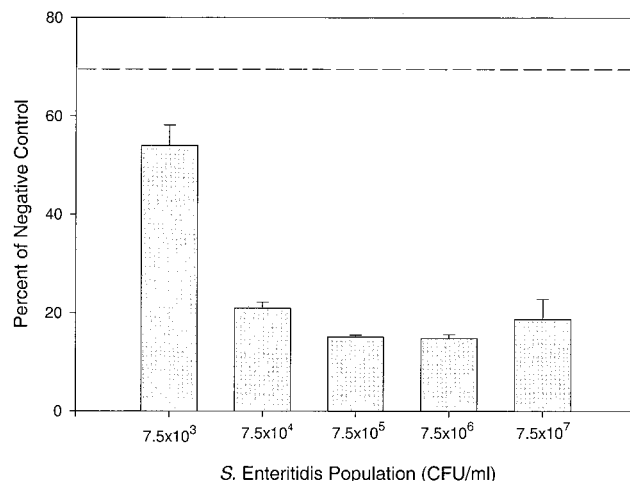


FIG. 4. Sensitivity of the IMS-bacteriophage assay as determined with five populations of *Salmonella* serovar Enteritidis. Values are expressed as percentages of the mean negative control value. The assay end point was determined by using fluorescence measurements. The dashed line indicates the 70% cutoff value for positive tests. The error bars indicate 1 standard deviation.

timal concentration for the assay, as removal of phage would presumably be more complete if a lower initial phage concentration was used.

(iii) Media. None of the alternative media tested performed as well as the L broth for supporting the growth of SACs in the absence of phage or the reduction of SACs by phage. A washing step to remove broth from the sample tubes is necessary when fluorescence is used as an end point.

Freeze-dried SACs. Because of the potential problems with maintaining and manipulating live *Salmonella* stock cultures in an industrial food lab, the use of a freeze-dried SAC population was investigated as an alternative to the use of an overnight culture. A comparison of the titers of samples before and after freeze-drying indicated there was approximately a 30%

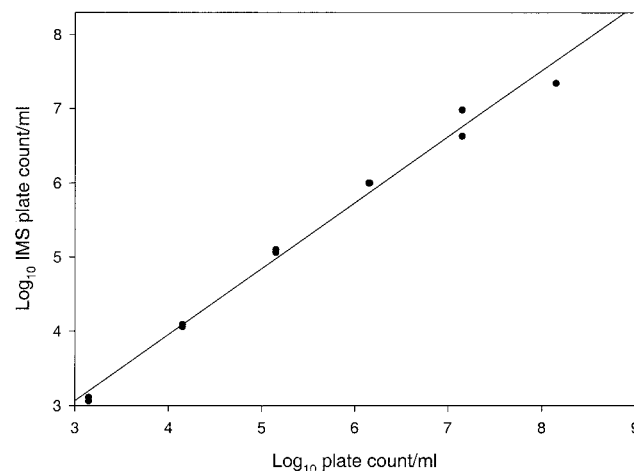


FIG. 5. Relationship between plate counts and plate counts obtained following IMS for different dilutions of *Salmonella* serovar Enteritidis in broth. Two replicates from each 10-fold dilution (10^3 , 10^4 , 10^5 , 10^6 , 10^7 , and 10^8 CFU/ml) were counted. The line is the fitted regression line ($y = 0.89x + 0.39$; $r^2 = 0.99$).

loss of viability as a result of the freeze-drying process. Duplicate samples prior to freeze-drying had bacterial titers of 1.7×10^8 and 1.9×10^8 CFU/ml. The counts obtained for duplicate samples after freeze-drying were 1.3×10^8 and 1.2×10^8 CFU/ml.

The lag phase of freeze-dried cultures was also investigated. Samples were reconstituted in L broth, and growth, as determined by an increase in optical density, was observed within 1 h. Freeze-dried SACs were compared with an overnight SAC culture by using the IMS-bacteriophage assay. Freeze-dried samples were reconstituted at the beginning of the protocol and incubated until they were required for the assay (approximately 2.5 h). The optical densities at 600 nm of the freeze-dried cultures reached about 0.060 during this time. An overnight SAC culture was adjusted to the same optical density. The sensitivity of the assay was the same whether the SACs were freeze-dried or derived from an overnight culture (Fig. 3), indicating that freeze-dried cells were a suitable alternative to an overnight culture.

DISCUSSION

The procedure for detection and isolation of *Salmonella* serovar Enteritidis described here is rapid, sensitive, specific, and easy to perform. The assay can be completed in 4 to 5 h. The IMS-bacteriophage assay is based on isolation of target cells by using paramagnetic beads coated with polyclonal antibodies against *Salmonella* and subsequent infection of the target cells by a lytic bacteriophage. The resulting progeny phage are recovered and assayed by using SACs. In negative samples, the SACs grow in the absence of phage. In positive samples, growth of the SACs is slowed compared to growth in the negative samples, and over time the population may decrease. This difference can be quantified by using optical density or fluorescence measurements.

The results of the specificity study indicate that bacteriophage SJ2 was not specific for *Salmonella* serovar Enteritidis, nor was its host range sufficient for a genetic *Salmonella* test (Table 1). The current method, therefore, can be used as a model system. All five *Salmonella* serovar Enteritidis isolates tested, as well as the 15 *Salmonella* serovar Enteritidis outbreak isolates, were positive as determined by both the IMS-bacteriophage assay and the plaque assay. Strong positive results were also observed with *S. enterica* serovar Sendai (group D) and *Salmonella* serovar Typhimurium SA 942256. This *Salmonella* serovar Typhimurium strain was confirmed by serology analysis to belong to group B. *Salmonella* serovar Typhimurium 94-51 was not sensitive to the phage. Two other serotypes belonging to serogroup D, *S. enterica* serotype Berta and *S. enterica* serotype Panama, produced somewhat inconsistent results with both the plaque assay and the IMS-bacteriophage assay. The repetitive O-antigen structures of *Salmonella* strains are known to be receptors for *Salmonella* phage, such as phage P22 of *Salmonella* serovar Typhimurium (24). Positive strains had the same diagnostically important somatic (O) or flagellar (H) antigens as strains found to be uninfected by phage SJ2. Somatic antigen 09 was found in all of the serogroup D *Salmonella* strains tested, but this antigen is not present in *Salmonella* serovar Typhimurium. O antigens 01 and 12 are found in all of the strains that test positive but are also

found in other group B strains (2). Outer membrane proteins are also known to be receptors for phage in gram-negative organisms, including phage lambda of *Escherichia coli* K-12 (27). Further work will be necessary to determine the receptor(s) for phage SJ2. A more suitable choice for a generic *Salmonella* test may be R-core-specific bacteriophage Felix-O1 (19), which was shown to infect more than 98% of the more than 5,200 *Salmonella* strains tested (9).

In the early development of the assay, prior to the use of IMS, centrifugation was the technique used to separate unbound phage from the samples after the attachment step. This procedure was successful for tests of pure cultures in broth but did not address the problem of competitive flora and food matrix effects that could be encountered in food sample enrichments. In order to make the assay more applicable to eventual testing of food samples, IMS was employed. Magnetic particle separation is important not only for initial recovery of target cells but also for permitting subsequent washing steps (phage removal) and for immobilizing the beads and cells for recovery of progeny phage in the supernatant.

There was good correlation between IMS and plate counts over the range of cell concentrations tested ($r = 0.99$, $P < 0.001$). At the 10^{-6} dilution (1.4×10^3 CFU/ml), 86% of the bacteria were recovered. The level of recovery dropped to 15.7% for the 10^{-1} dilution (1.4×10^8 CFU/ml), probably because of the increased cell-to-bead ratio. There were approximately 1.3×10^7 beads added per ml of sample (Dynal Inc., personal communication). At the highest dilution, this equaled a cell-to-bead ratio of about 10 to 1. Vermunt et al. (31) reported a recovery rate of $51\% \pm 7.8\%$ following IMS, although these authors prepared beads by using a noncommercial source of antibodies.

The one-step growth experiment performed with bacteriophage SJ2 was important in that it determined some of the incubation times required for the assay. Since lysis began to occur after 32 min (Fig. 2), the length of the attachment and washing steps could not exceed this time; otherwise, progeny phage might have been lost. As a result, 10 min was used for attachment of phage, followed by 20 min for washing. The results of the growth experiment suggest that a 30-min incubation period for amplification would be sufficient for one complete round of infection.

The optimal parameters for the assay were determined to be an assay temperature of 37°C and a phage population size of 10^7 PFU/100 μ l, and L broth was the most suitable medium for SAC incubation (although any nonselective nutrient medium that supports growth of *Salmonella* should be suitable).

Fluorescent probes, such as rhodamine 123, carbocyanine, calcafluor white, oxonol dye, and ethidium bromide, have been used to determine bacterial viability, with different degrees of success (17, 18, 25). A novel LIVE/DEAD bacterial viability kit has been developed by Molecular Probes, and this kit has two fluorescent components: a live stain, SYTO 9, and a dead stain, propidium iodide (Molecular Probes product information). SYTO 9 is a cell-permeating nucleic acid stain that fluoresces green in all cells when it is excited by blue light. Propidium iodide is a nucleic acid stain that does not permeate cells and stains only cells with compromised membranes. Propidium iodide competes for binding sites with SYTO 9 in such cells, and the result is cells that stain red when they are excited by blue light.

Bogosian et al. (4) evaluated the viable-but-non-culturable state in *E. coli* and concluded that cell membrane integrity is not a suitable predictor of cell viability. They developed a mixed-culture recovery method which demonstrated that the apparently nonculturable cells were not viable but not culturable but were in fact dead, despite the fact that they continued to stain green with the Molecular Probes LIVE/DEAD BacLight viability stain throughout the 300-day experiment.

In the present study cell death occurred because of lysis of the cells by phage; thus, the LIVE/DEAD stain is an appropriate choice to monitor viable cell numbers. Preliminary work with a modified direct epifluorescent filter technique (23) indicated there was good correlation between plate counts and direct epifluorescent filter technique counts when the LIVE/DEAD stain was used (data not shown). When measurements were determined with a fluorometer, L broth was found to interfere with the fluorescent signal, resulting in high background levels. Lambda buffer and lambda buffer supplemented with one of two sugars (dextrose or maltose) at three concentrations (0.2, 0.5, and 1.0%) were evaluated for the ability to support growth of SACs in the absence of phage, as well as the ability to provide a suitable environment for reduction of SACs by phage. These media did not interfere with fluorescent staining but did not perform nearly as well as L broth for both parameters.

Although the initial analyses in which fluorescence was used as the end point for the IMS-bacteriophage assay were successful, it became clear that positive and negative samples could be distinguished visually based on the turbidities of the samples. Optical density was then examined as a way to determine the assay end point. Using optical density has other advantages. First, a spectrophotometer is a relatively inexpensive and common piece of laboratory equipment. Second, sample manipulation and total costs are reduced, as there is no need for fluorescent staining. Third, sample results can be read directly after removal from the incubator, without the need for a washing step to remove media. And fourth, the assay is flexible, since samples can be reincubated after initial readings are obtained to further distinguish between positive and negative samples. The use of fluorescence as an end point has none of these advantages.

The limit of detection of the IMS-bacteriophage assay in broth was determined to be less than 10^4 CFU/ml (Fig. 3). The sample values were calculated as percentages of the mean negative control value. A sample value of 70% or less of the mean negative control value was considered positive. The 70% cutoff value was determined based on preliminary work performed with artificially inoculated food samples and gave reliable discrimination between positive and negative samples. Hirsch and Martin (11) reported on another *Salmonella* detection assay based on the principle of assaying progeny phage. They described a method for detection of *Salmonella* by using bacteriophage Felix-O1 and high-performance liquid chromatography. The interaction of bacteriophage Felix-O1 with *Salmonella* serovar Typhimurium resulted in increased numbers of bacteriophage which were subsequently detected by high-performance liquid chromatography. The detection limit was determined to be 10^6 *Salmonella* cells per ml (10). Stewart et al. (29) described a similar method termed the phage amplification assay. In this method, phage were incubated with target cells, and unbound phage were destroyed by a novel virucidal

agent that did not destroy the target cells. Progeny phage were then assayed by a plaque assay with the aid of a helper population of bacteria. The detection limit was reported to be 40 bacteria per ml for *Pseudomonas aeruginosa* and 600 bacteria per ml for *Salmonella* serovar Typhimurium (29). Considering legislative requirements that a rapid method for detecting *Salmonella* spp. must be able to detect one viable bacterial cell per 25 g of food sample, some form of preenrichment would still be required to reach the reported detection limit of 600 *Salmonella* serovar Typhimurium cells per ml. Also, Stewart et al. employed only pure cultures. The problem of competitive microflora and the possible interference of phage binding by food matrices that could be encountered in food sample enrichments would likely compromise the assay sensitivity and necessitate a selective enrichment or separation step.

The BIND test (Idetek Corp.) is the only commercially available bacteriophage assay for detection of *Salmonella* in food and is based on production of ice nuclei after infection by phage (33). A sensitivity of 10 CFU/ml has been reported for this assay, and food material was found not to interfere with the assay (32). The BIND assay was compared with the Reveal ELISA test (Neogen Corp.), a filter monitor test from Future Medical Technologies, and a standard plating method for detection of *Salmonella* in fresh and frozen poultry and environmental samples (22). Peplow et al. concluded that the BIND assay, in its current state of development, is not suitable for routine monitoring of environmental samples due to its low sensitivity, which results in many false-negative results.

The detection limits of ELISAs have been reported to range from 10^4 to 10^8 CFU/ml (6, 13, 21). The IMS-bacteriophage assay has a detection limit comparable to or better than those of all of the other available detection methods, except the gene amplification methods.

One of the problems common with antibody-based tests is nonspecific binding. Parmar et al. (20) evaluated an IMS-conductance method for detection of *Salmonella* in milk powders. In evaluating the specificity of IMS by using anti-*Salmonella* Dynabeads, they noted that exposure to *Citrobacter freundii* resulted in a *Salmonella* type of conductance curve and concluded that the magnetic beads were only partially specific for the *Salmonella* strains tested. Nonspecific binding of *C. freundii* has been reported by other authors (6). In contrast, because of the increased specificity of the IMS-bacteriophage assay imparted by the bacteriophage, nonspecific binding should not lead to false-positive results and should not interfere with the assay provided an appropriate number of *Salmonella* cells can be recovered on the beads. The *C. freundii* included as part of the phage specificity test in this study did not result in a positive test. Factors that affect immunological and nonspecific binding to magnetic beads include the culture medium, the bacterial strain, and the particle-cell incubation time and temperature (3).

Maintaining and propagating live *Salmonella* cultures for use as SACs in the assay may not be desirable in an industrial food laboratory. Freeze-dried *Salmonella* cultures that were reconstituted on the day of testing were investigated as an alternative to overnight cultures. The sensitivity of the assay was not compromised when freeze-dried SACs were used (Fig. 3). A format could be developed so that freeze-dried cells could be resuspended and incubated and the progeny phage

could be added with minimal risk of release into the lab environment. Another alternative would be the use of auxotrophic mutants or the use of avirulent mutants of phage host cultures.

A limitation of the protocol described here is that it is not suitable for routine screening of large numbers of samples. A maximum of 12 sample tubes can be comfortably assayed at one time. Microtiter plates coated with polyclonal antibodies against *Salmonella*, combined with recent developments in plate washers, could increase the number of samples that can be tested at one time and lead to partial automation of the protocol. Another improvement to the protocol may be immobilization of bacteriophage on magnetic beads or microtiter plates for capture and separation of target cells from food sample enrichments. Bennett et al. (1) described a novel biosorbent consisting of a *Salmonella*-specific bacteriophage passively immobilized on a solid phase, which included plastic dipsticks and microtiter plates. However, the capture efficiency was poor, possibly because of the orientation of the phage on the solid phase (attachment by both head and tail groups) or because of the small surface area of microtiter wells. Bennett et al. noted that further research would optimize biosorbent construction in terms of the type of phage used, the orientation of the phage, and the choice of solid phase. Use of a phage-based biosorbent in the present study would have reduced the total assay time and sample manipulation time by combining the capture and attachment steps. In addition, the high specificity of a phage for its target bacterium may reduce the nonspecific attachment often observed with antibody-based applications.

The IMS-bacteriophage assay described here is based on the combined specificity of immunomagnetic beads and bacteriophage SJ2 for *Salmonella* spp. Our results demonstrate that the normal biology of bacteriophage SJ2 in its host bacterium can be exploited and that progeny phage can subsequently be assayed indirectly by determining their effect on a helper population of healthy *Salmonella* serovar Enteritidis cells. The assay end point can be determined either by optical density or fluorescent measurements. The assay is not specific for *Salmonella* serovar Enteritidis, nor is it suitable for a generic *Salmonella* test, but it provides a good working model for future development of the assay with other host-phage systems. The assay is simple to perform, can be completed in 4 to 5 h, and has a limit of detection of less than 10^4 CFU/ml. The next step in the development process will be to test the assay with artificially inoculated food samples.

ACKNOWLEDGMENTS

This work was supported by funds from Corvin Inc., Hamden, Conn. We especially thank Ted Heying of GEM Biomedical.

REFERENCES

- Bennett, A. R., F. G. Davids, S. Vlahodimou, J. G. Banks, and R. P. Betts. 1997. The use of bacteriophage-based systems for the separation and concentration of *Salmonella*. *J. Appl. Microbiol.* **83**:259–265.
- Bergey, D. H., J. G. Holt, N. R. Krieg, and P. Sneath (ed.). 1984. *Bergey's manual of systematic bacteriology*, vol. I. Williams and Wilkins, Baltimore, Md.
- Blackburn, C. D. W. 1993. Rapid and alternative methods for the detection of salmonellas in foods. *J. Appl. Bacteriol.* **75**:199–214.
- Bogosian, G., P. J. L. Morris, and J. P. O'Neil. 1998. A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. *Appl. Environ. Microbiol.* **64**:1736–1742.
- Chen, J., and M. W. Griffiths. 1996. *Salmonella* detection in eggs using *lux*⁺ bacteriophages. *J. Food Prot.* **59**:908–914.
- Cudjoe, K. S., T. Hagtvædt, and R. Dainty. 1995. Immunomagnetic separation of *Salmonella* from foods and their detection using immunomagnetic particle (IMP)-ELISA. *Int. J. Food Microbiol.* **27**:11–25.
- Dubow, M. S. 1994. Bacterial identification—use of bacteriophages, p. 78–81. In R. G. Webster and A. Granoff (ed.), *Encyclopedia of virology*. Academic Press, San Diego, Calif.
- Fluit, A. C., M. N. Widjoatmodjo, A. T. A. Box, R. Torensma, and J. Verhoef. 1993. Rapid detection of salmonellae in poultry with the magnetic immuno-polymerase chain reaction assay. *Appl. Environ. Microbiol.* **59**:1342–1346.
- Gunnarsson, A., B. Hurrell, and E. Thal. 1977. Recent experiences with the *Salmonella*-O1 phage in routine diagnostic work. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.* **237**:222–227.
- Hirsch, D. W., and L. D. Martin. 1983. Rapid detection of *Salmonella* spp. by using Felix-O1 bacteriophage and high-performance liquid chromatography. *Appl. Environ. Microbiol.* **45**:260–264.
- Hirsch, D. W., and L. D. Martin. 1983. Detection of *Salmonella* spp. in milk by using Felix-O1 bacteriophage and high-performance liquid chromatography. *Appl. Environ. Microbiol.* **46**:1243–1245.
- Holt, P. S., R. K. Gast, and C. R. Greene. 1995. Rapid detection of *Salmonella enteritidis* in pooled liquid egg samples using a magnetic bead-ELISA system. *J. Food Prot.* **58**:967–972.
- June, G. A., P. A. Sherrod, and W. H. Andrews. 1992. Comparison of two enzyme immunoassays for the recovery of *Salmonella* spp. from four low-moisture foods. *J. Food Prot.* **55**:601–604.
- Kodikara, C. P., H. H. Crew, and G. S. A. B. Stewart. 1991. Near on-line detection of enteric bacteria using *lux* recombinant bacteriophage. *FEMS Microbiol. Lett.* **83**:261–266.
- Mansfield, L. P., and S. J. Forsythe. 1993. Immunomagnetic separation as an alternative to enrichment broths for *Salmonella* detection. *Lett. Appl. Microbiol.* **16**:122–125.
- Maramorosh, K., and H. Koprowski. 1967. *Methods in virology*. Academic Press, New York, N.Y.
- Mason, D. J., R. López-Amoros, R. Allman, J. M. Stark, and D. Lloyd. 1995. The ability of membrane potential dyes and calcein white to distinguish between viable and non-viable bacteria. *J. Appl. Bacteriol.* **78**:309–315.
- Matsuyama, T. 1984. Staining of living bacteria with rhodamine 123. *FEMS Microbiol. Lett.* **21**:153–157.
- McConnell, M. R., and J. E. Schoelz. 1983. Evidence for shorter than average O-polysaccharide chainlength in the lipopolysaccharide of a bacteriophage Felix-O1 sensitive variant of *Salmonella anatum* A. 1. *J. Gen. Microbiol.* **129**:3177–3184.
- Parmar, N., M. C. Easter, and S. J. Forsythe. 1992. The detection of *Salmonella enteritidis* and *Salmonella typhimurium* using immunomagnetic separation and conductance microbiology. *Lett. Appl. Microbiol.* **15**:175–178.
- Patel, P. D., and D. W. Williams. 1994. Evaluation of commercial kits and instruments for the detection of foodborne bacterial pathogens and toxins, p. 60–103. In P. D. Patel (ed.), *Rapid analysis techniques in food microbiology*. Chapman & Hall, Glasgow, Scotland.
- Peplow, M. O., M. Correa-Prisant, M. E. Stebbins, F. Jones, and P. Davies. 1999. Sensitivity, specificity, and predictive values of three *Salmonella* rapid detection kits using fresh and frozen poultry environmental samples versus those of standard plating. *Appl. Environ. Microbiol.* **65**:1055–1060.
- Pettipher, G. L. 1983. The direct epifluorescent technique for the rapid enumeration of microorganisms. Research Studies Press Ltd., London, United Kingdom.
- Potete, A. R. 1994. P22 bacteriophage, p. 1009–1003. In R. G. Webster and A. Granoff (ed.), *Encyclopedia of virology*. Academic Press, San Diego, Calif.
- Puchkov, E. O., and A. N. Melkozerov. 1995. Fluorimetric assessment of *Pseudomonas fluorescens* after freeze-thawing using ethidium bromide. *Lett. Appl. Microbiol.* **21**:368–372.
- Scheffe, H. 1963. *The analysis of variance*. John Wiley & Sons Inc., New York, N.Y.
- Schwartz, M., and L. Le Minor. 1975. Occurrence of the bacteriophage lambda receptor in some *Enterobacteriaceae*. *J. Virol.* **15**:697–685.
- Stewart, G. S. A. B. 1990. *In vivo* bioluminescence: new potentials for microbiology. *Lett. Appl. Microbiol.* **10**:1–8.
- Stewart, G. S. A. B., S. A. A. Jassim, S. P. Denyer, P. Newby, K. Linley, and V. K. Dhir. 1998. The specific and sensitive detection of bacterial pathogens within 4 h using bacteriophage amplification. *J. Appl. Microbiol.* **84**:777–783.
- Swaminathan, B., and P. Feng. 1994. Rapid detection of food-borne pathogenic bacteria. *Annu. Rev. Microbiol.* **48**:401–426.
- Vermunt, A. E. M., A. A. J. M. Franken, and R. R. Beumer. 1992. Isolation of salmonellas by immunomagnetic separation. *J. Appl. Bacteriol.* **72**:112–118.
- Wolber, P. K., and R. L. Green. 1990. New rapid method for the detection of *Salmonella* in foods. *Trends Food Sci. Technol.* **1**:80–82.
- Worthy, W. 1990. Bacteria assay exploits ice nucleation research. *Chem. Eng. News* **68**:23–25.
- Yu, H., and P. J. Stopa. 1996. Application of an immunomagnetic assay system for detection of virulent bacteria in biological samples, p. 297–306. In J. M. Van Emon, C. L. Gerlach, and J. C. Johnson (ed.), *Environmental immunochemical methods: perspectives and application*. American Chemical Society, Washington, D.C.